Final Report

Project Title: Probiotics to Increase Shellfish Hatchery Production

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Saltonstall-Kennedy Program
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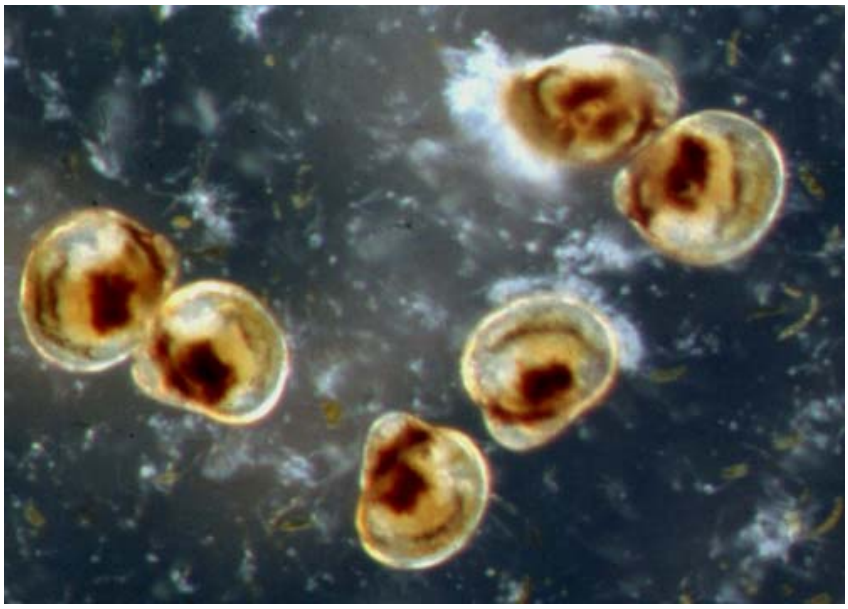

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I. Abstract

The purpose of the project was to identify and test the approach of using probiotic bacteria to prevent bacterial diseases in shellfish hatcheries. The laboratory validation of the beneficial effect of specific probiotic bacterial species was the goal of this project and consisted of three objectives: (1) identify and quantify probiotic activity of candidate species of bacteria, (2) confirm identity of the bacterial species and (3) conduct laboratory tests of probiotic effectiveness to prevent bacterial disease in shellfish larvae and seed. We characterized both laboratory archived bacterial strains as well as 24 new isolates for probiotic activity. Significant probiotic activity was found. The identity of bacterial isolates and a portion of the bacterial pathogenicity testing for the project was performed as a University of Washington Master's thesis research project conducted by Ms. Robyn Estes (Estes 2002). This thesis work consisted of characterization of 109 bacterial isolates, pathogenicity testing of a selection of the isolates and phenotypic and phylogenetic analysis of larval oyster pathogens and related bacterial cultures. The 16s rDNA of eight strains of bacteria was cloned and sequenced in order to evaluate the phylogenetic relationship between these strains and known and described species of bacteria.

To complete objective three of the project, three highly pathogenic isolates were confirmed and selected for both *in vitro* and *in vivo* evaluation and for challenging larval Pacific oysters and geoduck clams with and without probiotics. Several of the strong probiotic producers were pathogenic to the shellfish larvae and others exhibited very slow growth rates. Nonetheless, we identified one probiotic candidate that provided partial but significant protection to larval oysters and clams challenged with pathogenic bacteria. We also isolated additional useful strains of non-pathogenic and rapidly growing bacteria with probiotic activity (*in vitro*) near the end of this project but did not have resources to test these for their ability to protect larval or juvenile shellfish from bacterial challenge. The results indicate that successful probiotic candidates must be non-pathogenic and rapidly proliferating in order to exclude pathogens from larval cultures.

III. Executive Summary

The purpose of the project was to identify possible probiotic bacteria and test the approach of using them to prevent bacterial diseases in shellfish hatcheries. Adding beneficial species of bacteria to shellfish hatchery systems may exclude disease causing bacteria. Previous research has shown that certain strains of marine bacteria produce natural antibiotics that inhibit the production of disease causing bacteria but the concept has not yet been implemented in commercial hatcheries.

Pathogenic and probiotic bacteria were characterized and then tested in a laboratory microcosm apparatus to determine if a protective effect could be observed when the larval or juvenile oysters or clams were exposed to pathogens. The laboratory validation of the beneficial effect of specific probiotic species was the goal of this project and consisted of three objectives: (1) identify and quantify probiotic activity of candidate species of bacteria, (2) confirm identity of the bacterial species and (3) conduct laboratory tests of probiotic effectiveness to prevent bacterial disease in shellfish larvae and seed.

We characterized both laboratory archived bacterial strains as well as 24 new isolates for probiotic activity. Significant probiotic activity was found. While we proceeded to test these isolates for pathogen inhibitory activity *in vivo*, as described under Objective 3 of the report, we continued to search for probiotic candidates during the course of the project.

The identity of bacterial isolates and a portion of the bacterial pathogenicity testing for the project was performed as a University of Washington Master's thesis research project conducted by Ms. Robyn Estes (Estes 2002). This thesis work consisted of (1) characterization of 109 bacterial isolates using fatty acid analysis and restriction fragment length polymorphisms, (2) pathogenicity testing of a selection of the isolates, and (3) phenotypic and phylogenetic analysis of larval oyster pathogens and related bacterial cultures. This work demonstrated the characteristics of pathogenic and non-pathogenic strains of bacteria and the groupings (relatedness) of over 100 strains of shellfish hatchery bacteria using principal component analysis. The 16s rDNA of eight strains of bacteria was cloned and sequenced in order to evaluate the phylogenetic relationship

between these strains and known and described species of bacteria. The number and descriptive detail of isolates characterized by Estes (2002) exceeded that which we expected to characterize in the initial project plan.

To complete objective three of the project, we confirmed and utilized the pathogenicity of three bacterial pathogens for oyster and clam larvae for both *in vitro* and *in vivo* testing. Several of the strong probiotic producers were pathogenic to the shellfish larvae and others exhibited very slow growth rates. Nonetheless, we identified one probiotic candidate that provided partial but significant protection to larval oysters and clams challenged with pathogenic bacteria. The results indicate that successful probiotic candidates must be non-pathogenic and rapidly proliferating in order to exclude pathogens from larval cultures.

The results also demonstrated that shellfish pathogens themselves may produce probiotic substances and this may, in fact, be a key factor by which they exclude other bacteria and proliferate in the larval and juvenile shellfish cultures, to the exclusion of other benign or beneficial bacteria. Rapid colonization of shellfish cultures with pathogens must be overcome with probiotics that themselves replicate rapidly or are established in the shellfish cultures prior to the introduction of pathogens.

We isolated additional bacterial strains with probiotic activity (*in vitro*) in the last quarter of the project. Some of these strains proliferated rapidly on Marine Agar at temperatures of between 15°C and 25°C. We did not have time to fully test these for their ability to protect larval shellfish from bacterial pathogens but expect to do so in a continuing project.

This project advanced the objective of obtaining probiotic bacteria for shellfish culture. A large number of probiotic producers were identified and both these and pathogens were characterized. The research demonstrated that many probiotic producers are mildly to strongly pathogenic and that many grow slowly under laboratory conditions. One promising probiotic candidate was characterized and more than 10 additional rapidly growing isolates with probiotic activity were obtained but not fully tested. The exclusion of pathogens will likely be both a result of inhibitory substances secreted by other bacteria as well as rapid growth and competition that excludes pathogens and occupies their ecological niche in the culture system.

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IV. Purpose

The purpose of the project was to identify and test the approach of using probiotic bacteria for preventing bacterial diseases in shellfish hatcheries. Adding beneficial local species of bacteria to shellfish hatchery systems may exclude disease causing bacteria. Beneficial bacteria have been used in various forms of animal husbandry for many years (Fuller 1992). One beneficial attribute of certain bacteria is the production of natural antibiotics that exclude disease causing bacteria. These natural substances are known as bacteriocin-like inhibitory substances (BLIS) or probiotics. Previous research has shown that certain strains of beneficial marine bacteria produce natural antibiotics that inhibit the production of disease causing bacteria and could be extremely useful in shellfish hatcheries (Austin et al. 1995; Bergh 1995; Gibson et al. 1998; Riquelme et al. 1997; Ruiz et al. 1996). However, the concept has not yet been successfully demonstrated and implemented in commercial hatcheries (Samocha et al. 1998).

Using a collection of bacteria from shellfish hatcheries and other sources, as well as new isolates, we selected those with the strongest probiotic effect when tested against our laboratory collection of bacteria pathogenic to shellfish larvae. Bacteria were characterized and then tested in a laboratory microcosm apparatus to determine if a probiotic or protective effect could be observed when the larvae or seed were exposed to pathogens. The laboratory validation of the beneficial effect of specific probiotic species was the goal of this project.

Inadequacy of existing methods for disease prevention in shellfish hatcheries. A number of bacterial species, typically of the *Vibrio* or closely related groups, have long been known to cause rapidly fatal diseases in oyster and other bivalve larvae and seed (Elston 1999). While some of the disease causing species have been identified, others apparently are undescribed species. Control of diseases in bivalve husbandry requires both sanitation methods and health management methods. Methods such as the use of ultraviolet sterilization (Brown 1979) for the disinfection of incoming sea water and other management methods can assist in reducing the bacterial load in the husbandry system (Elston 1999). In spite of the implementation of these approaches, pathogenic bacteria

still enter the husbandry systems and cause significant disease. Resulting losses can be severe, especially in the case of valuable single seed shellfish. Antibiotics have been used experimentally since the beginning of bivalve husbandry to control bacterial diseases (Walne 1958). However, there is legitimate concern about the development of antibiotic resistant bacteria. Due to public health and environmental concerns in the United States, registration of new antibiotics for use in shellfish husbandry is extremely expensive, if not cost prohibitive, and use of such drugs carries a potential risk of negative perception by the consuming public. Therefore, the use of probiotic bacteria as a natural supplement to the hatchery culture system appears to offer an effective and safe alternative for the control of bacterial diseases.

V. Approach

A. Description of Work Performed. The approach to the project, which consisted of three objectives and testable hypotheses is summarized in Table 1.

Table 1. Summary of project technical objectives, testable hypotheses and methods.

Project Objective	**Testable Hypothesis**	**Method Used**
1. Identify and quantify probiotic activity of candidate species of bacteria.	Significant probiotic activity exists in locally available marine bacteria.	Plate screen method for probiotic activity (Gibson et al. 1998).
2. Confirm identity of both probiotic species and west coast hatchery pathogens.	Pathogenic bacteria and probiotic producing bacteria are either established or undescribed species.	Biochemical methods, nucleic acid sequencing and sequence comparison, and cellular fatty acid determination.
3. Laboratory test of probiotic effectiveness to prevent bacterial disease in shellfish larvae and seed.	Probiotic producing bacterial species will prevent bacterial disease in shellfish seed and larvae under controlled conditions.	Laboratory bioassay method using oyster and clam larvae and seed.

The methods used to attempt the completion of these three objectives and the work performed are summarized as follows:

1. Identify and quantify probiotic activity of bacteria.

Based on our preliminary screening of bacterial strains and published reports of marine bacteria with useful probiotic activity (Austin et al. 1995, Bergh 1996, Gibson et al. 1998; Riquelme et al 1997, Ruiz et al. 1996), a small percentage of beneficial marine bacteria produce natural antibiotics that can inhibit the growth of disease causing bacteria. Isolates archived from shellfish diagnostic cases as well as new isolates from a variety of sources were used to establish which isolates had either the presence of probiotic activity or pathogenicity. The archived isolates consisted of approximately 150 isolates used in salt tolerance tests and those obtained from marine sediments, plants, shellfish and digestive tracts of marine fish, as well isolates representing several distinct shellfish pathogens. The low-passage bacterial isolates were stored at –70°C and recovered as needed for characterization or experimental work.

A relatively simple but effective assay as described by Gibson et al (1998) was used to screen bacteria for probiotic production. This is a modification of the deferred, diametric streak technique of Mayr-Harting et al. (1972) in which the probiotic producer is grown in tryptic soy broth (sodium supplemented for marine bacteria) and inoculated from the broth in a streak across tryptic soy agar (sodium supplemented) as a 0.5 cm streak. After incubation, the bulk of the resulting colonies are aseptically scraped away without damaging the agar surface and the remaining cells killed by exposure to chloroform fumes for 30 minutes. After dissipation of residual chloroform, the plates are streaked with shellfish pathogens at right angles to the original growth line of the potential probiotic producer. After incubation, the plates are examined and measured for a zone of growth inhibition in relation to the original growth footprint. Provided that uniformity in the method is carefully practiced, a relative degree of probiotic activity can be measured.

Based on the criteria used by Gibson et al. (1998), an inhibition zone of 10 mm or greater around the original streak occurred with a strong probiotic producer.

2. Characterize bacterial species.

The characteristics that determine the nature and ultimately the identity of over 100 marine bacterial species were established. This information was expected to be useful in differentiating pathogens from probiotic producers and provide data regarding the maintenance and growth of bacterial isolates. Additionally, we suspected that some of the most important shellfish pathogens in our collection are not currently designated species.

We initially planned to perform biochemical and physical characterization of bacteria in our laboratory and to subcontract fatty acid analysis and nucleic acid analysis out to a commercial laboratory. However, during the project, we modified this approach. The modification was approved by the project sponsor. Rather than subcontracting the characterization of bacteria to a commercial laboratory, we established a collaboration with Dr. Russ Herwig of the School of Fisheries and Ocean Sciences, University of Washington. Through this collaboration, a Master of Science degree was completed by Ms. Robyn Estes. In addition to Dr. Herwig as the Chairperson of this thesis committee for Ms. Estes, Drs. Faye Dong, Ralph Elston and Carolyn Friedman served as advisors on the committee. The subject of this thesis project was the biochemical and nucleic acid characterization of the 109 isolates of marine bacteria that we provided as well as testing a selection of the bacteria for pathogenicity. The methods utilized are described in detail in the completed thesis which is entitled: Characterization of pathogenic and non-pathogenic bacteria associated with bivalve mollusk larvae and shellfish hatcheries in the Pacific Northwest and Hawaii (Estes, R. M., 2002, University of Washington). The use of fatty acid analysis, analysis of restriction fragment length polymorphisms, and 16S ribosomal RNA gene sequence comparison were primary methods of characterization as well as more routine but important phenotypic and biochemical characterizations.

3. Laboratory microcosm evaluation of probiotic effectiveness.

Early in the project we identified several strong probiotic producers when tested against our isolated shellfish pathogens from hatcheries in west coast states. These isolates were tested for pathogenicity to oyster and geoduck larvae and their growth rate under typical temperature conditions found in shellfish hatcheries. Subsequently, the probiotic strains were tested for their ability to prevent or moderate disease caused by the pathogenic bacteria. We used an established laboratory assay system in which we inoculate cultures of shellfish larvae and seed with disease causing bacteria. Comparable cultures also have the putative probiotic strains added. We then determined the percentage reduction in morbidity and mortality to assess probiotic effectiveness in the microcosm shellfish cultures.

The laboratory method used for assaying the virulence of shellfish hatchery bacteria and probiotic effectiveness using both larval shellfish and seed shellfish up to 2 mm shell height is described in (Table 2). We tested pathogens and probiotic candidates on both Pacific oyster (*Crassostrea gigas*) and geoduck clam (*Panope abrupta*) larvae.

Table 2. Target experimental parameters for laboratory testing of probiotic effectiveness to prevent bacterial disease in shellfish seed and larvae.

Experimental Parameter	Larval Tests	Juvenile Tests
Species used	*C. gigas* (Cg), *P. abrupta* (Pa)[1]	Cg, Pa
Age or size	mid-veliger stage	1 mm to 2 mm shell height (Cg) or shell length (Pa)
Volume, dimension of test containers	100 mm petri dishes, 30 mL seawater	100 mm petri dishes, 30 mL seawater
Test animal density	5 per mL	5 per mL
Temperature of test	Cg: 23°C +/- 2°C Pa: 15°C +/- 2°C	Cg: 23°C +/- 2°C Pa: 15°C +/- 2°C
Water change frequency	24 hours	24 hours
Feeding	None	At 72 hours for 144 hour test, none for 96 hours test

Experimental Parameter	Larval Tests	Juvenile Tests
Method of inoculation	Concentrated washed cells scraped from Marine agar plate. Inoculate once only at beginning of test[2]	Concentrated washed cells scraped from Marine agar plate. Inoculate once only at beginning of test[2]
Inoculum concentration or range of concentrations	Typical test range is 1 x10^2 to 1 x 10^4 cells per mL for virulent isolates.	Typical test range is 1 x10^2 to 1 x 10^4 cells per mL for virulent isolates.
Replication	None for initial dose range finding assay. Definitive tests typically require three replicates[3]	None for initial dose range finding assay. Definitive tests typically require three replicates[3]
Statistical evaluation	Analysis of variance (ANOVA)	ANOVA
Frequency of morbidity, mortality enumeration	Every 24 hours	Every 24 hours
Histological evaluation	All dead individuals during test pooled. All individuals alive at end of test examined.	All dead individuals during test pooled. All individuals alive at end of test examined.

[1]*Crassostrea gigas* (Cg), Pacific oysters; *Panope abrupta* (Pa), geoduck clams.
[2]Pathogens and probiotic candidates will be grown on Marine Agar or other media, if required. Log phase cultures will be scraped from plates and washed in sterile seawater (SSW). We will establish the relationship of spectrophotometric absorbance (620 nm) to culture concentration in SSW prior to inoculation.
[3]Replication required will be assessed based on variance of initial assays and power analysis.

B. Project Management. Dr. Ralph Elston was the project manager and responsible for project coordination and conduct of the laboratory scale challenge assays. Dr. Gee was responsible for isolating and characterizing candidate probiotic bacteria. Drs. Herwig and Elston were responsible for the supervision of the thesis work conducted by Ms. Robyn Estes, along with Drs. Faye Dong and Carolyn Friedman. This thesis work consisted of the characterization of bacteria contained in Objective 2 of the project.

VI. Findings

A. Actual accomplishments and findings.

Objective 1: Identify and quantify probiotic activity of bacteria.

We characterized laboratory archived and new isolates for probiotic activity during the course of this project. New isolates obtained with probiotic activity are shown in Table 3.

Table 3. New isolates obtained during this project with variable degrees of probiotic activity.

Source of Putative Probiotic Bacteria	Number of Putative Probiotic Strains
Asian salted fish source	4
Oyster gut	8
Aquarium treatment product	2
Salmon gut	1
Marine algal cultures	1
Crab digestive organ	1
Sea water debris from oyster and clam beds	5
Sea water from Seattle Aquarium	2

These isolates were tested using the cross streak method (Gibson et al 1998). An example of a plate inoculated with a putative probiotic strain of bacteria is shown in Fig. 1. After the test strain of bacterial growth is obtained, as shown in Fig. 1, the bacterial cells are scraped from the plate and residual bacteria on the plate is killed with chloroform vapor. The residue containing the potential probiotic substance is contained between the two lines drawn vertically on the plate. An example of three degrees of pathogen inhibition are shown in Figs. 2 and 3. In Fig. 2, two strains of pathogenic bacteria were tested, represented by the horizontal lines across the plate. The upper streak of bacteria shows moderate inhibition of the test pathogen while the lower streak of bacteria representing a second pathogen shows no inhibition of the pathogenic strain. Fig. 3 shows two strains of pathogenic bacteria tested against the probiotic candidate. In this case, growth of both

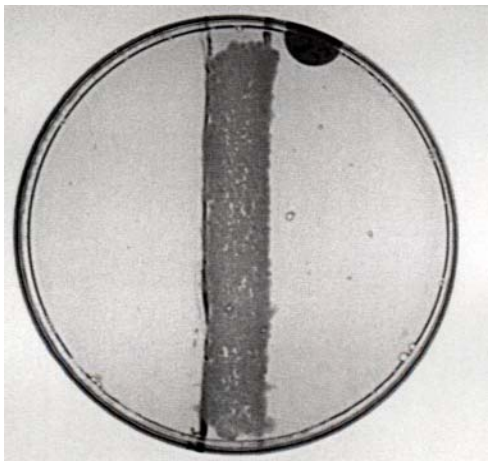


Fig. 1. Marine agar bacterial plate streaked with putative probiotic strain of bacteria. The bacterial growth is the dark vertical streak between two lines drawn on the plate.

pathogens was inhibited by the candidate probiotic strain. After establishing that the 24 new strains of probiotic candidates and the additional archived strains of probiotic candidates had some degree of probiotic activity, they were further tested for pathogenicity and growth rate as described in the results of objective 3 of the report.

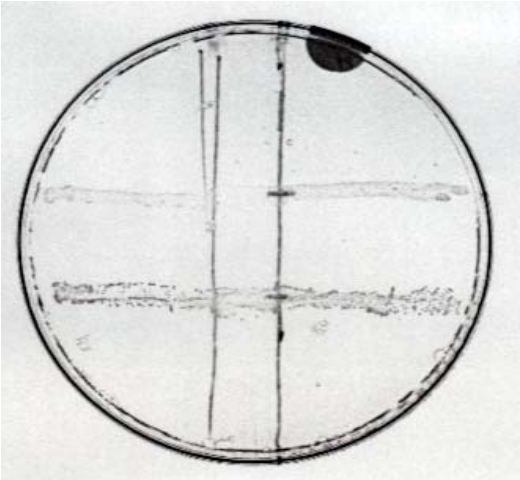


Fig.2. Marine agar bacterial plate streaked with probiotic candidate G-9 and two pathogens (upper and lower horizontal streaks). The upper horizontal streak (pathogen A) is moderately inhibited in the probiotic zone while the lower pathogen streak (pathogen B) shows no inhibition by the probiotic.

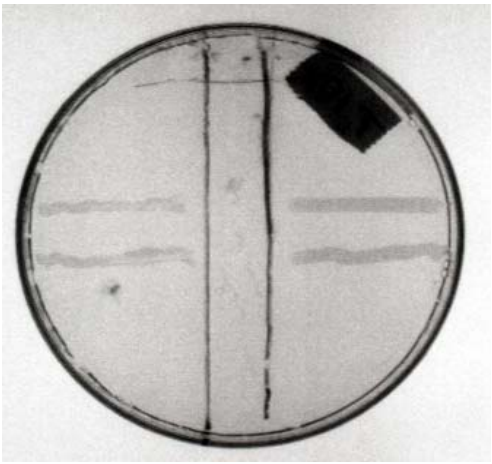


Fig. 3. Marine agar bacterial plate streaked with probiotic candidate O-1 and two pathogens (upper and lower horizontal streaks). Both pathogen A and B (upper and lower horizontal streaks, respectively) show marked inhibition by probiotic strain O-1.

Some strains of probiotic candidates were eliminated from further testing as described below but those that were selected for further testing are shown in Table 4. These strains were used to determine if they provided protection to oyster and clam larvae challenged with pathogenic bacteria.

Table 4. Summary of inhibition of three larval shellfish pathogens (A,B and C) by eight probiotic candidate bacteria, using the agar streak inhibition method.

	Bacterial Isolate Designation							
	G9	G10	G12	O1	O3	Cy9	Cy10	S13
Inhibition of test pathogen A[1]	S	S	S	S	M	S	S	S
Inhibition of test pathogen B	0	M	M	S	S	M	M	S
Inhibition of test pathogen C	M	M	S	S	S	M	M	M

[1] Inhibition code: S – strong; M – moderate; 0 – no inhibition

We proceeded to test these isolates for pathogen inhibitory activity *in vivo*, as described under Objective 3 of this report. However, we continued to search for probiotic candidates during the course of the project and found additional promising candidates including several that produced shellfish pathogen inhibitory substances, had growth rates that approximated those of the pathogens (i.e. rapidly proliferated in Marine Agar or Marine Broth when grown at 20°C to 25°C) and were non-pathogenic in initial tests.

Objective 2. Characterize bacterial species

The characterization of bacterial isolates and a portion of the bacterial pathogenicity testing for the project was performed as a part of the University of Washington Master of Science thesis research project conducted by Ms. Robyn Estes (Estes 2002). A brief summary of these results will be presented here from the Estes (2002) thesis. This thesis work consisted of (1) characterization of 109 bacterial isolates using fatty acid analysis and restriction fragment length polymorphisms; (2) pathogenicity testing of a selection of the isolates and (3) phenotypic and phylogentic analysis of larval oyster pathogens and related bacterial cultures.

Figure 4 shows a summary dendrogram from Estes (2002) that demonstrates that 116 shellfish bacterial isolates (archived and new isolates), and reference strains, were separated into five groups, based on fatty acid analysis. The dendrogram is expanded into further figures showing the relatedness of members of each group in the thesis (Estes 2002) and shows that many of the isolates grouped closely with species of *Vibrio*, *Pseudomonas*, and several other genera.

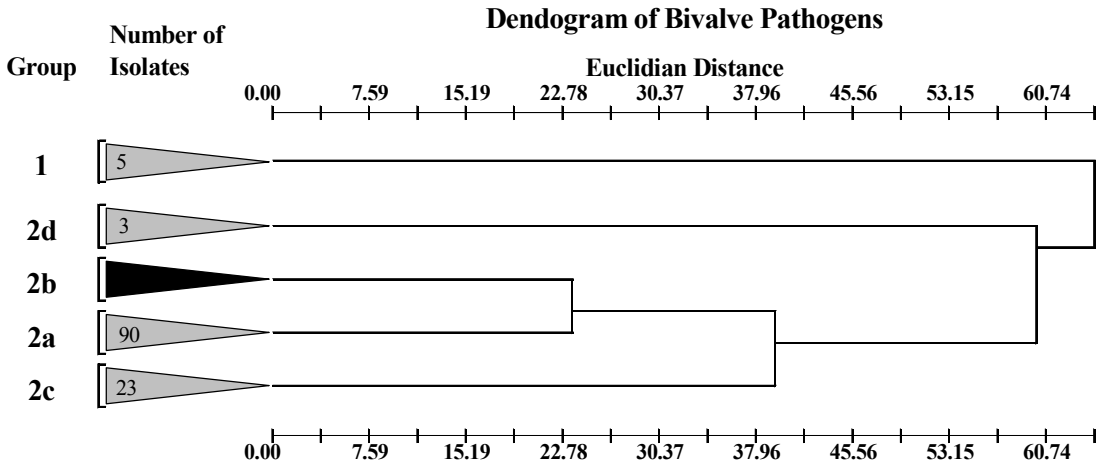


Fig. 4 (Figure 2.3, Estes 2002). Dendogram of fatty acid profiles of 116 bacterial isolates associated with bivalve larvae and shellfish hatcheries in the Pacific Northwest and Hawaii and 13 ATCC type strains. All groups are collapsed.

Estes (2002) further evaluated the relatedness of bacterial strains, based on fatty acid analysis using principle component analysis. The results of her principle component analysis are shown in Figs. 5 and 6 and show the how the total number of isolates fell into related groups, based on fatty acid analysis. The rationale for principal component analysis is discussed in the thesis (Estes 2002).

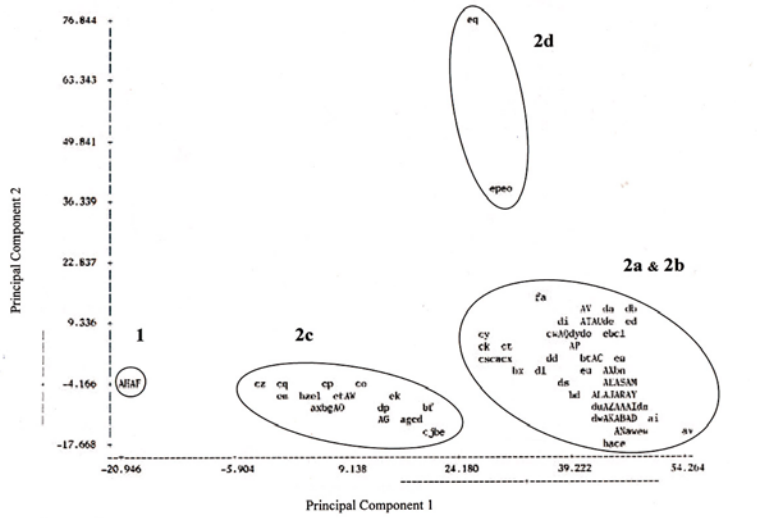


Fig. 5 (Figure 2.8, Estes 2002). 2-D plot of principal components of fatty acid profiles of shellfish hatchery and control bacterial strains.

Estes (2002) also used restriction fragment length polymorphisms (RFLP) to characterize the shellfish bacterial isolates. This analysis can be found in her thesis, along with further details of the fatty acid analysis. She concluded that the fatty acid analysis was more efficient for characterizing large populations of bacteria and more sensitive to subtle differences between the isolates.

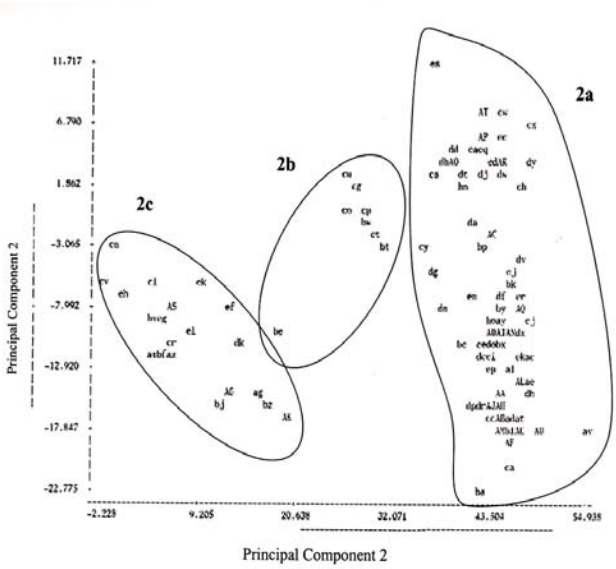


Fig. 6 (Figure 2.9, Estes 2002). 2-D plot of principal components of fatty acid profiles of shellfish hatchery and known isolates. Gram positive isolates (group 1: RE 8, RE 9, RE 14, RE 39, and RE 85) and outlying isolates (Group 2d: RE 115, RE 117, and RE 118) were omitted.

Estes (2002) tested the pathogenicity to larval Pacific oysters of 29 of the bacterial isolates. These results are discussed in detail in the thesis. The pathogenicity testing of Estes (2002) confirmed that several isolates were highly pathogenic to larval oysters and these were included in the pathogens used to test for probiotic activitiy *in vitro*.

Estes (2002) also cloned and sequenced the 16s rDNA of larval oyster pathogens and related non-pathogenic isolates in order to evaluate the phylogenetic relationship between these strains and known and described species of bacteria. She sequenced eight of the 109 strains that were provided from the archived collection and also completed phenotypic testing and phylogenetic analysis. The strains that were characterized consisted of three highly pathogenic strains, one mildly pathogenic strain and four non-pathogenic strains isolated from similar locations and environments as the pathogenic strains. Phylogenetic analysis was performed by comparing the 16s rDNA sequences obtained from the eight isolates to the data contained in the Ribosomal Database Project

II using similarity ranking and sequence alignment software. Estes (2002) found that the 16S rDNA sequences were all very similar to *Vibrio* species as shown in Fig. 7.

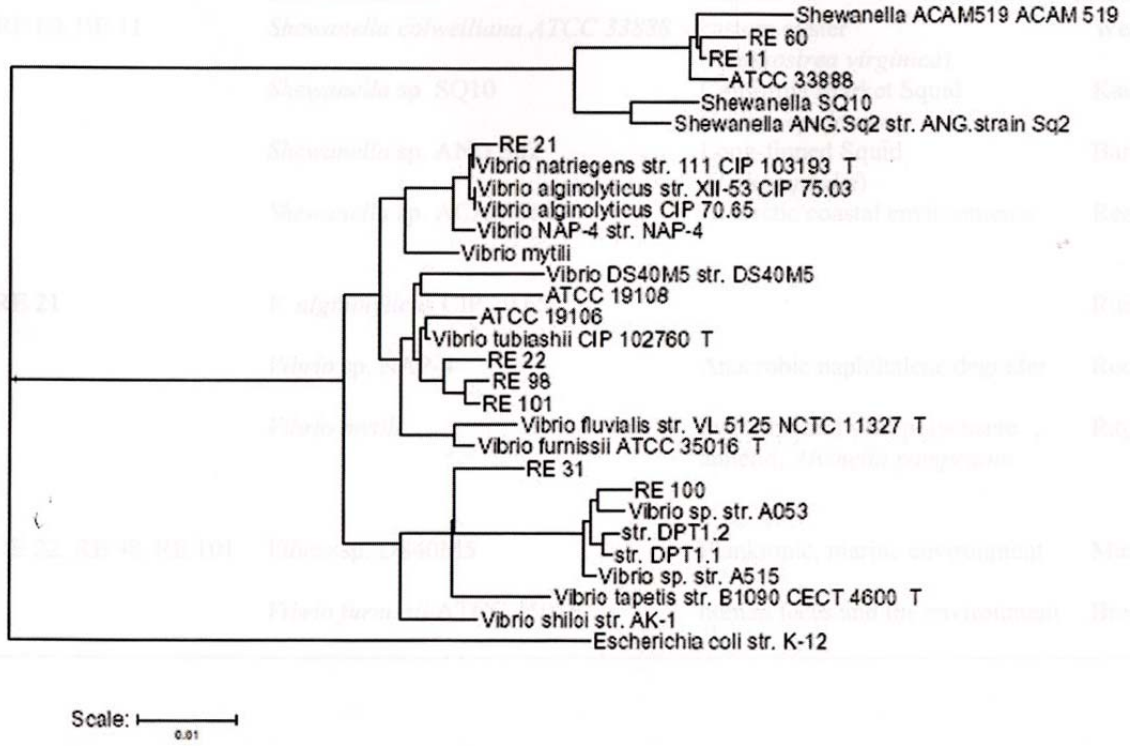


Fig. 7 (Figure 4.1, Estes 2002). Phylogenetic analysis of 16s rDNA sequences of isolates collected from larval Pacific oysters and closely related strains present at the Ribosomal Database Project. Strains with an "RE" designation represent those isolated from shellfish hatcheries and provided for characterization by R. Elston. Bar represents 1 nucleotide change per 100 bases.

The 109 bacterial isolates characterized by Estes (2002) exceeded the number of isolates we expected to characterize in the initial project plan. In fact, the work contained in the Estes (2002) thesis expanded the objectives of bacterial characterization significantly. This was appropriate since we discovered after the beginning of the project that the number of shellfish bacterial isolates, their pathogenicity and probiotic activity were more complex than originally envisioned, including the fact that isolates with probiotic activity may also be pathogenic. Thus, we determined that a basic step in advancing the objective of probiotic development for shellfish hatcheries was to better characterize both pathogens and non-pathogenic strains of bacteria.

In addition to the characterization of shellfish hatchery bacteria performed by Robyn Estes for her thesis research, the fatty acid profiles of probiotic candidates were determined to establish the likely identifications and relationships of these bacterial to pathogens and bacteria in general. Most of the probiotic candidates fell into the *Vibrio* or related groups. Based on the fatty acid profiles, the relatedness of the probiotic candidates fell into 7 groups represented in the dendrogram in Fig. 8.

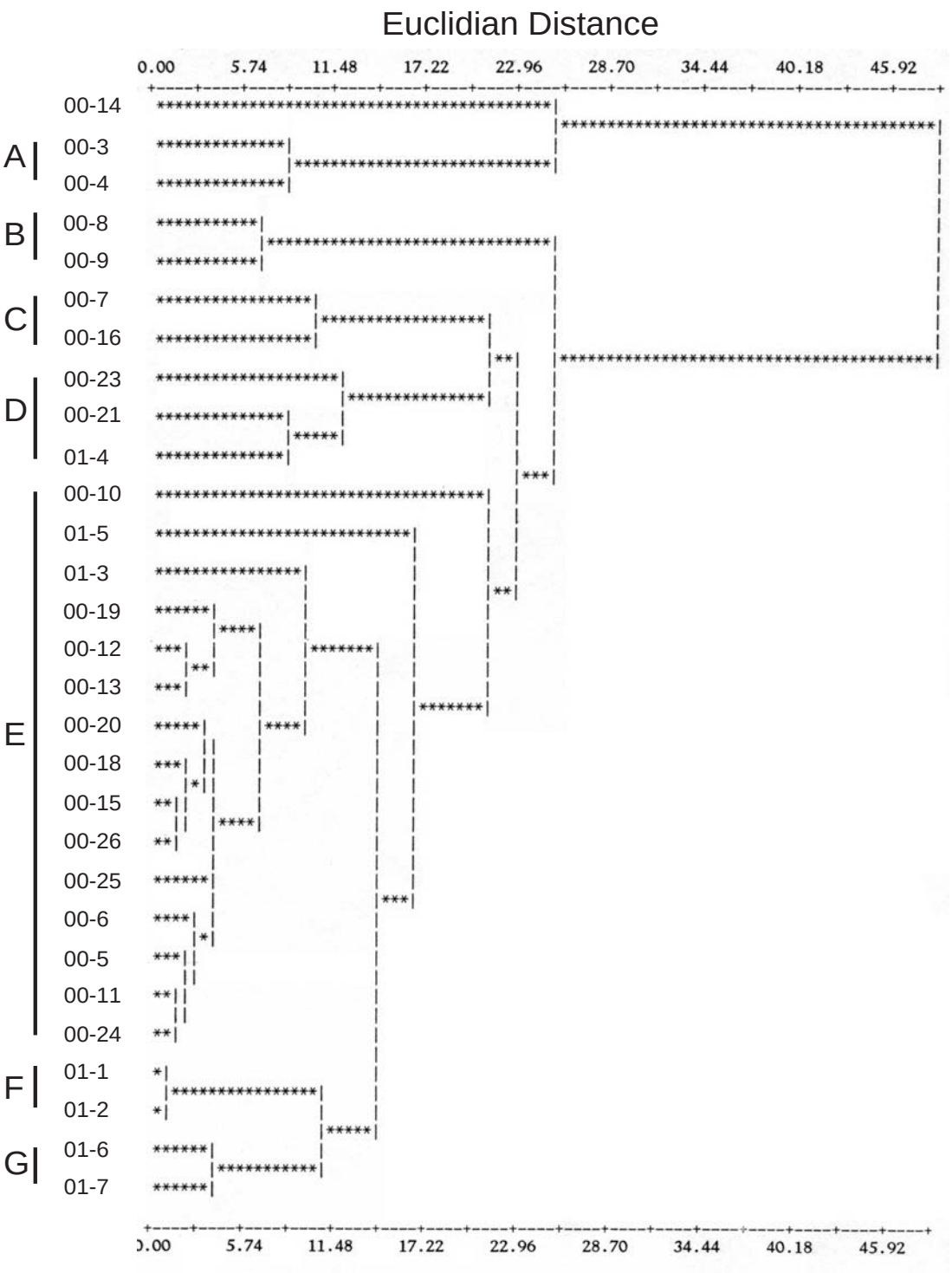


Fig. 8. Dendrogram showing grouping of probiotic candidates based on fatty acid analysis of 29 probiotic producing isolates.

Objective 3. Laboratory microcosm evaluation of probiotic effectiveness.

Selection of pathogens and confirmation of pathogenicity. We used the laboratory pathogens X00-12-1 (=pathogen A), 00-90-1 (=Pathogen B) and 99-70-6B-2 (Pathogen C) for testing probiotic protection, based on our confirmation of their pathogenicity (Fig. 9) and the tests of pathogenicity conducted in the Estes (2002) thesis. The methods for larval pathogenicity tests are given in Table 2 of this report. To evaluate the tests, the number of swimming larvae and the number of alive and dead larvae was estimated by examining the cultures with a stereomicroscope at 24 and 48 hours. The larvae exposed to the pathogenic isolates displayed typical bacterial swarming associated with massive bacterial infections in the terminal stages of the disease. In the early observations larvae were inactive and this inactivity was followed by an increase of the number of motile bacteria observed in the cultures, including the formation of bacterial swarms near the larvae (Figs. 10a and 10b).

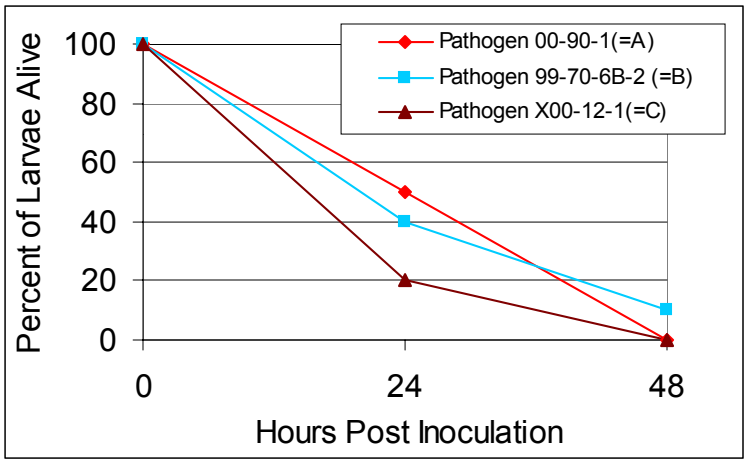
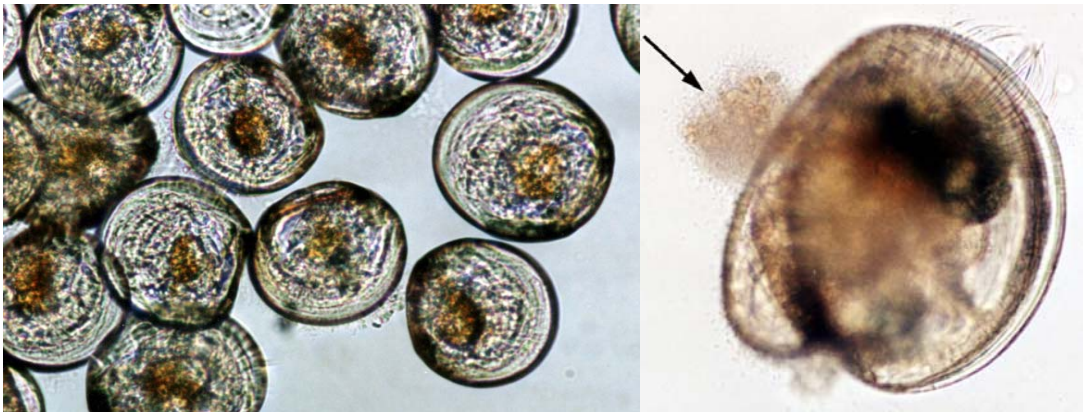


Fig. 9. Confirmation of pathogenicity of bacterial isolates A, B and C for oyster larvae (*Crassostrea gigas*). Each point represents the average of three values. The pathogen target dose was 1 x 10^5 bacterial per mL. A similar set of pathogenicity curves was generated for these same isolates for geoduck larvae (*Panope abrupta*).



Figs. 10a (left) and 10b (right). Figure 10a shows inactive early umbo larvae a few hours after inoculation of bacterial pathogen B. Figure 10b shows typical bacterial swarming (arrow) in an oyster larvae with a late stage bacterial infection.

Evaluation of probiotic candidates for pathogenicity to oyster larvae and geoduck larvae. Prior to testing the efficacy of probiotic candidates for protection of shellfish larvae and juveniles, the probiotic candidates themselves needed to be tested for pathogenicity to the shellfish. In order to accomplish this objective, we performed standard pathogenicity assays with probiotic candidates. The probiotic candidates were grown on marine agar and resuspended in sterile seawater so that their concentration

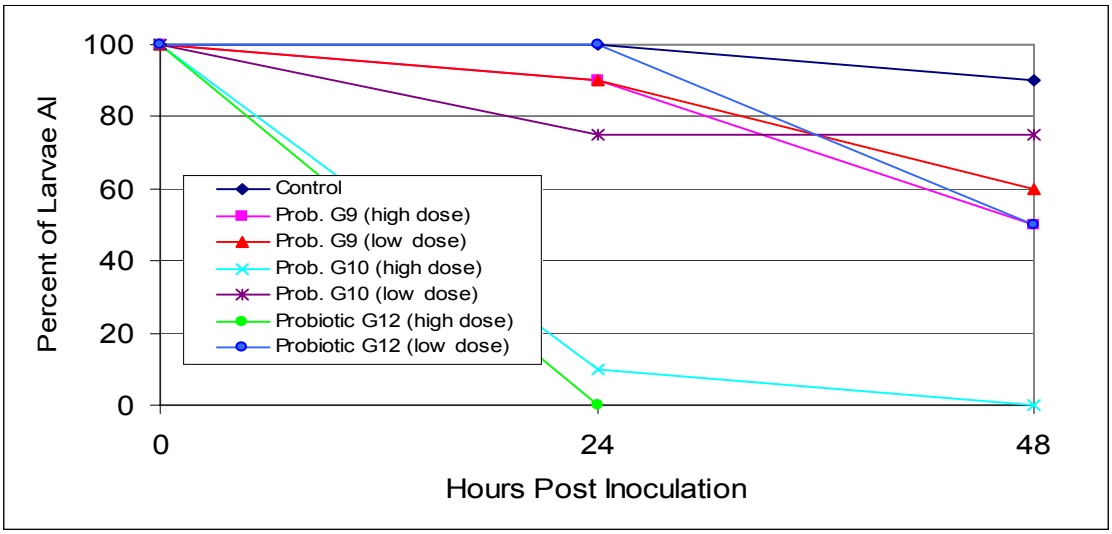


Fig. 11. Challenge of oyster larvae with probiotic candidates at a high dose (10^5 cfu per mL) and a low dose (10^3 cfu per mL). Results of the challenge (% of live oyster larvae were recorded at 24 and 48 hours. Each point represents the average of three replicate values.

could be determined and uniform inoculations made. The probiotic candidates were inoculated at two target concentrations range of 1 x 10^3 and 1 x 10^5 cfu per mL. Results typical of a group of probiotic candidates found to be moderately to highly pathogenic to oyster larvae are shown in Fig. 11.

During the testing of probiotic candidates, a second group that did not show pathogenicity to either geoduck or oyster larvae was identified. Typical challenge results

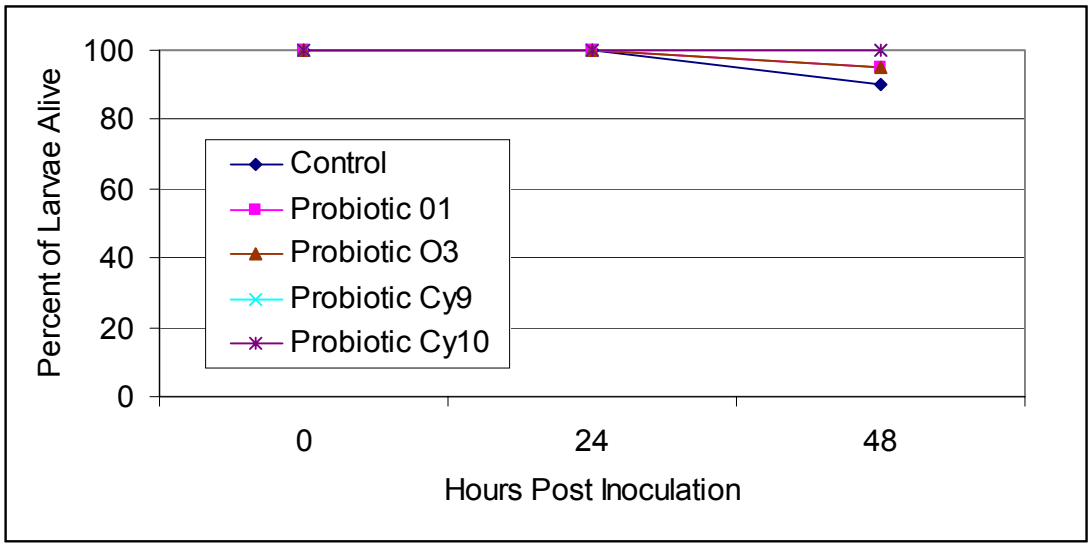


Fig. 12. Challenge of oyster larvae with probiotic candidates at a high dose (10^5 cfu per mL) of bacterial pathogen B. Results of the challenge (% of live oyster larvae were recorded at 24 and 48 hours. Each point represents the average of three replicate values. The results for probiotic candidates O1 and O3 and Cy9 and Cy10 are merged and not distinguished in the graph.

are shown in Fig. 12. Overall, seven probiotic producing isolates were found that were not pathogenic to oyster or clam larvae, even at doses as high as 1 x 10^6 bacteria per mL.

Evaluation of growth rate of probiotic candidates. After establishing that some of the probiotic candidates from the first group of archived and new groups were non-pathogenic to oyster and geoduck larvae, we tested their growth rates on laboratory medium in comparison with pathogens. Results were fairly uniform in that probiotic

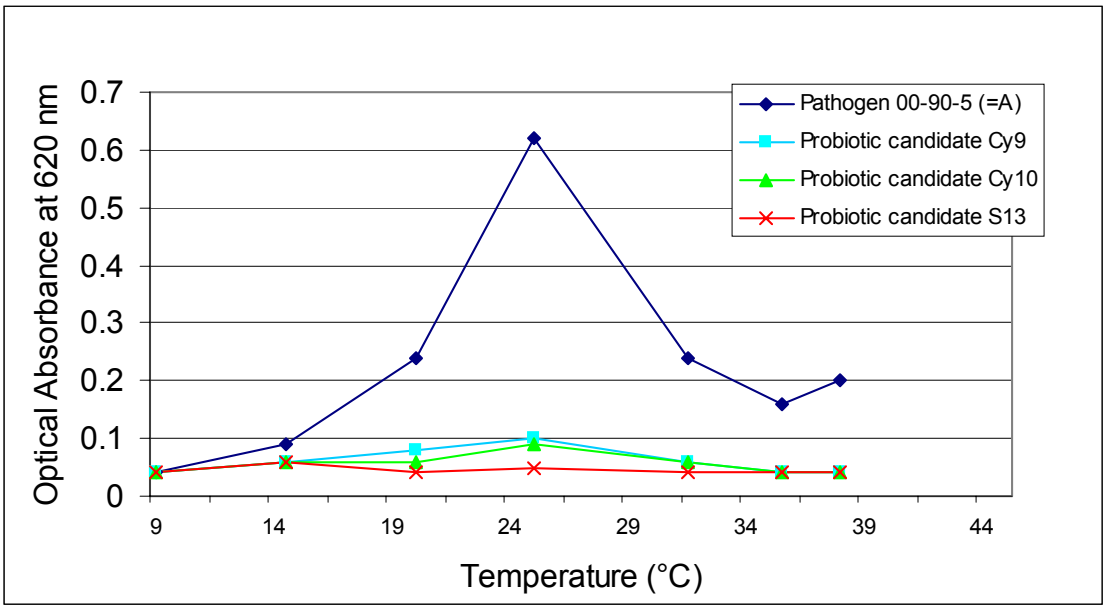


Fig. 13. Comparison of growth rate of Pathogen A in comparison to growth rate of three probiotic candidates at temperatures from about 9°C to about 39°C. Tests were conducted in Marine Broth.

candidates showed a relatively slow growth rate in comparison to pathogens (Fig. 13). This slow growth of probiotic candidates proved to later complicate the testing procedure. In most cases, it required 6 to 8 days to obtain harvestable quantities of bacteria that could be used in the *in vivo* assays.

Evaluation of probiotic efficacy in laboratory microcosms. According to the previously defined protocol, we set up experiments to test the combined addition of oyster and geoduck pathogens with non-pathogenic probiotic candidates. The probiotic candidates were added to larval cultures about 1 hour prior to the addition of pathogens. Probiotic candidates were added at an estimated concentration range of between 1 x 10^4 to 1 x 10^5 cfu per mL. The concentration that could be added was limited by the slow growth and consequently minimized harvests of the probiotic candidates.

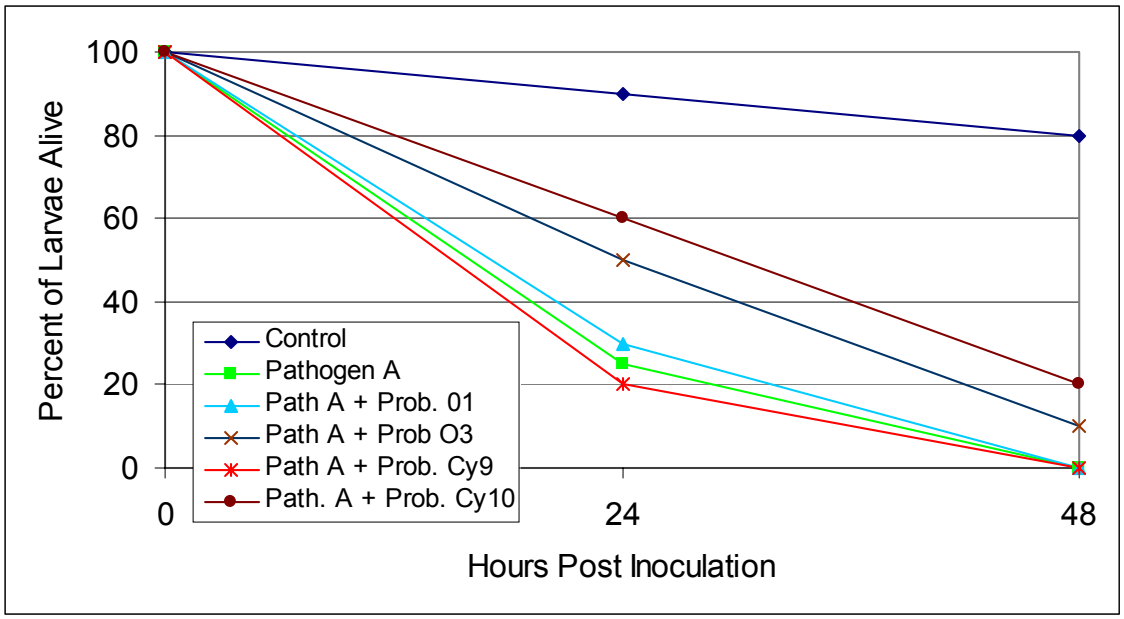


Fig. 14. Results of experiment to show the effects of adding probiotic candidates to larval oyster cultures that received Pathogen A. Each point on the graph represents the average of three replicate cultures.

Figure 14 shows results that are typical of those we obtained when testing larval oysters and geoducks with candidate probiotic strains of bacteria, in the presence of oyster pathogens. Most probiotic treatments appeared to have little effect. However, probiotic candidate Cy10 was significantly different from the pathogen A only treatment for both oyster larvae and geoducks at 24 hours post inoculation but not at 48 hours post-inoculation. However, it was also significantly different from the uninoculated control as well at both 24 and 48 hours. This result suggests some intermediate degree of protection. One primary reason that we may not have attained a higher degree of protection with Cy10 and any protection at all with other isolates tested was their slow growth rate under laboratory conditions, assuming this equates to slow growth in larval cultures as well.

Isolation of additional probiotic candidate bacteria. In addition to the probiotic candidates we tested in this project, represented in the foregoing examples, we also isolated additional strains with probiotic activity (*in vitro*) in the latter part of the project. Some of these strains proliferated rapidly on Marine Agar at temperatures of between

15°C and 25°C and were not pathogenic to oyster or clam larvae in preliminary assays. Due to limited time and resources, we were not able to fully test these for their ability to protect larval shellfish from bacterial pathogens but expect to do so in a continuing project.

B. Discussion and Conclusions – Problems with Probiotic Activity of Candidates

Utility of the tested probiotic candidates. While the results with the archived strains of bacteria and new probiotic candidates (Table 3) indicated that some probiotic candidates were also pathogens, many had very slow growth rates and only a few apparently have useful probiotic activity. This probiotic activity may potentially be optimized if the growth rate of the probiotic strains, such as Cy10 can be optimized and higher concentrations of the strain used to pre-condition larval cultures before the inoculation of bacterial pathogens.

Additional evaluation of larval and juvenile shellfish response to pathogens and probiotic candidates. While this project advanced the objective of obtaining probiotic bacteria for shellfish culture, it did not achieve the expected results for the *in vivo* tests, primarly due to the slow growth response and pathogenicity of some probiotic candidates. However, the bacterial strains obtained in the last quarter of the project appear to have greater potential due to their rapid growth rate and will be tested further after the completion of this project.

C. Need for additional work

Approach to probiotic additions to larval and juvenile shellfish cultures and additional needed research. In addition to providing shellfish cultures with beneficial bacteria that produce bacterial inhibitory substances, it also is likely that an approach to beneficial bacterial replacement that includes rapidly growing beneficial or benign bacteria is important, even if such benign bacteria do not produce probiotic substances. The exclusion of pathogens may be both a result of inhibitory substances secreted by other bacteria as well as rapid growth and competition that excludes pathogens or

occupies their ecological niche in the culture system. These factors will need to be taken into account in follow on work with the objective of obtaining effective shellfish hatchery probiotics.

References Cited

Austin, B., Stuckey, L. F., Robertson, P.A.W., Effendi, I., Griffith, D.R.W. 1995. A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordali*. Journal of Fish Diseases 18: 93-96.

Bergh, Ø. 1995. Bacteria associated with early life stages of halibut, *Hippoglossus hipploglossus* L., inhibit growth of a pathogenic Vibrio sp. Journal of Fish Diseases 18: 31-40.

Brown, C. 1979. Ultraviolet light: an effective disinfectant for shellfish hatcheries? Ozonews (part 2) February 1979, pp. 6-7.

Elston, R. A. 1999. Development, Histology and Health Management of Seed Oysters. 110 pages. World Aquaculture Society, Baton Rouge, Louisiana.

Estes, R. M. 2002. Characterization of pathogenic and non-pathogenic bacteria associated with bivalve mollusk larvae and shellfish hatcheries in the Pacific Northwest and Hawaii. Master of Science thesis, University of Washington.

Fuller, R. 1992. Probiotics, The Scientific Basis. Chapman and Hall, London.

Gibson, L. F., Woodworth, J., George, A. M. 1998. Probiotic activity *of Aeromonas media* on the Pacific oyster, *Crassostrea gigas*, when challenged with *Vibrio tubiashi*. Aquaculture 169: 111-120.

Jukes, T. H. and C. R. Cantor. 1969. Evolution of protein molecules, P. 21-132. In H. N. Munro (ed.). Mammalian protein metabolism. Academic Press, New York.

Mayr-Harting, A., Hedges, A. J., Berkeley, R. C. W. 1972. Methods for studying bacteriocins. Methods Microbiol. 7A: 325-422.

Ndowora, T.C.R., L.L. Kinkel, R.K.Jones, and N.A.Anderson. 1996. Fatty acid analysis of pathogenic and suppressive strains of *Stretomyces* species isolated in Minnesota. American Phytopathol. Soc. 86: 138-143.

Riquelme, C., Araya, R., Vergera, N., Rojas, A., Guanita, M., Candia, M. 1997. Potential probiotic strains in the culture of the Chilean scallop, *Argopecten purpuratus* (Lamarck, 1819). Aquaculture 154: 17-26.

Ruiz, C. M., Roman, G, Sánchez, J. L. 1996. A marine bacterial strain effective in producing antagonisms of other bacteria. Aquaculture International 4: 289-291.

Saitoun N., and M. Nei. 1987. The neighbor joining method: a new method for construction of phylogenetic trees. Mol. Biol. Evol. 4:406-425.

Samocha, T. M., Lawrence, A. L., Horowitz, A., and Horowitz, S. 1998. The use of commerical probiotics in the production of marine shrimp under no water exchange. Pages 373-375. Proceedings of the Second International Conference on Recirculating Aquaculture, July 16-18, 1998. Charleston, Virginia.

Walne, P. R. 1958. The importance of bacteria in laboratory experiments on rearing the larvae of *Ostrea edulis* (L.O.). Journal of the Marine Biological Association of the United Kingdom 37:415–425.

VII. Evaluation

The extent to which the project goals were met and modifications to the original project plan are explained as follows.

Project objective 1 was fully met. For objective 1, we planned to identify and quantify probiotic activity of candidate species of bacteria. This objective was completed using the plate screening method for probiotic activity. While many of the isolates we characterized with probiotic activity turned out not to be useful in laboratory micocosm applications, we learned important new information about the relationship between pathogens and production of probiotic substances. Specifically, this project determined that probiotic producers often are pathogenic to shellfish larvae, particularly at higher doses. However, near the end of the project, we isolated several promising new probiotic candidates that appear not to be pathogenic yet grow rapidly enough under laboratory conditions that they may be useful in hatchery application.

Project objective 2 was slightly modified but overall, the work completed for objective 2 exceeded the initial research plan. The modification to project objective 2 was to

incorporate the Master of Science thesis project of Ms. Robyn Estes under the direction of Drs. Russel Herwig, Faye Dong, Ralph Elston and Carolyn Friedman into the project instead of contracting bacterial analysis out to a commercial laboratory. The educational requirements allowed most of the original objectives to be met and also required additional research to comprise a complete master's thesis project. Specifically, we obtained a much more detailed analysis of shellfish hatchery pathogens and non-pathogens as well as additional pathogenicity testing than was anticipated in the original project design.

Project objective 3 was not completed to the extent anticipated in the initial project proposal. We expected to find more rapidly proliferating probiotic candidates. In addition, the pathogenicity of certain candidates was not expected. As a result, the number of candidates available for testing was more limited than expected. However, one promising candidate was identified that shows significant protection of oyster and clam larvae at 24 hours post-pathogen inoculation. Due to the slow growth of the probiotic candidates we were not able to test them in early stage juvenile shellfish as originally planned.

In the latter part of the project, we isolated additional more promising probiotic candidates. Due to the timing of these isolations and the fact that most of the project funding had been expended at that time, we were not able to fully characterize the utility of these probiotic candidates. However, we have subsequently planned and initiated a new project which will benefit from these isolations and initial *in vitro* characterizations of probiotic activity made under this project.

Dissemination of project results. The results of the project will be made available, at either a meeting of the Pacific Coast Shellfish Growers Association or a Washington Sea Grant Shellfish Growers meeting. Copies of the final report will be distributed to shellfish hatchery operators in California, Oregon, Washington, Alaska and Hawaii.